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# Yeast

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**Research Article**

# Characterization of a gene encoding tRNA nucleotidyltransferase from *Candida glabrata*

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## Abstract

A gene encoding ATP (CTP):tRNA nucleotidyltransferase (EC2.7.7.25) was isolated from *Candida (Torulopsis) glabrata* by complementation in *Saccharomyces cerevisiae*. The predicted amino acid sequence of the protein revealed a large region with high sequence similarity to members of the Class II group of the nucleotidyltransferase superfamily and an N-terminal region characteristic of a mitochondrial targeting sequence. The essential role of the carboxylates within the conserved DXD and RRD motifs was confirmed by mutagenesis. *C. glabrata* strains bearing truncated *CCAI* genes that lacked sequences encoding the putative mitochondrial targeting peptide were unable to grow on non-fermentable carbon sources but were able to grow on a fermentable carbon source. These results suggest that, as in *S. cerevisiae*, the *C. glabrata* CCA-adding enzyme is a sorting isozyme that functions in multiple cellular compartments. Mapping of the 5'-ends of primary transcripts of *CCAI* revealed multiple transcription start sites located both upstream of and between two in-frame start codons. When the cells were cultured on a non-fermentable carbon source the longer transcripts appeared more abundant, suggesting that the choice of transcription start sites was influenced by carbon source. The shorter transcripts, which lacked sequences encoding the mitochondrial targeting information, were more predominant in cells grown on glucose. These observations suggest that expression of CCA-adding isozymes in *C. glabrata* may be regulated. The DNA sequence has been assigned GenBank Accession No. AF098803. Copyright © 2002 John Wiley & Sons, Ltd.

**Keywords:** tRNA nucleotidyltransferase; *Candida glabrata*; *CCAI*; sorting isozyme

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## Introduction

The enzyme ATP(CTP):tRNA nucleotidyltransferase (EC 2.7.7.25) catalyses the synthesis and repair of the 3'-terminal cytidine, cytidine and adenosine (CCA) sequence of tRNAs. For most tRNA nucleotidyltransferases that have been studied, addition of both CTP and ATP residues is accomplished by a single polypeptide. However, in some organisms CCA-addition requires the participation of distinct ATP- or CTP-adding polypeptides (Tomita and Weiner, 2001). Eubacterial and eukaryotic tRNA nucleotidyltransferases share a conserved 25 kDa amino-terminal domain (Shanmugam *et al.*, 1996; Yue *et al.*, 1996). Sequence

and secondary structure comparisons revealed that this domain contains the active site signature G[SG][LIVMFY]x R[GQ]x<sub>5,6</sub>D[LIVM][DE]-[CLIVMFY]<sub>3</sub> (Holm and Sander, 1995) of the nucleotidyltransferase superfamily (Martin and Keller, 1996; Yue *et al.*, 1996). The absence of the 25 kDa amino-terminal domain from the CCA-adding enzyme from *Sulfolobus* led Yue *et al.* (1996) to subdivide the nucleotidyltransferase superfamily into two classes. Class I enzymes share only a small region of homology that contains the active site signature of Holm and Sander (1995) and include archaeobacterial CCA-adding enzymes, eukaryotic poly(A) polymerase, DNA

polymerase- $\beta$  and other nucleotidyltransferases, such as uridylyltransferase. Class II enzymes, including eubacterial poly(A) polymerases and both eubacterial and eukaryotic CCA-adding enzymes, share the 25 kDa amino-terminal domain and have a variation of the active site signature that contains a longer  $\alpha$  helix.

Stepwise addition of the nucleotides to 3'-tRNA termini by tRNA nucleotidyltransferase occurs in the absence of a nucleic acid template (Deutscher, 1973). The phosphoryl-transfer reaction is believed (Yue *et al.*, 1996) to proceed by the two-metal ion mechanism (reviewed in Steitz, 1999) used by other polynucleotide polymerases. The crystal structures of DNA polymerase- $\beta$  (Sawaya *et al.*, 1994; Davies *et al.*, 1994; Pelletier *et al.*, 1994) revealed three carboxylates that coordinate two metal cations. Sequence alignments suggested that this catalytic triad was found in other members of the nucleotidyltransferase superfamily (Martin and Keller, 1996), with two of the carboxylates being found on aspartates in a DXD motif within the signature sequence. For several class I enzymes, the requirement for a third carboxylate, located within an RXD motif (Martin and Keller, 1996; Yue *et al.*, 1996), has been demonstrated *in vitro* (Date *et al.*, 1991; Menge *et al.*, 1995; Martin and Keller, 1996). For Class II enzymes the third carboxylate was proposed to be located within an RRD motif (Martin and Keller, 1996; Yue *et al.*, 1996). Mutational analysis of the Class II enzyme, eubacterial poly(A) polymerase I, confirmed *in vitro* the requirements for the aspartates of the DXD and RRD motifs as well as two other carboxylates within the 25 kDa conserved domain (Raynal and Carpousis, 1999). Recently, Seth *et al.* (2002) showed that in the *E. coli* CCA-adding enzyme (a Class II enzyme), substitution of the aspartates in the DXD motif with alanines abolished C and A addition, while mutation of the aspartate in the RXD motif to alanine did not alter C addition but impaired A addition, suggesting a role for the third carboxylate in ATP binding. This observation is consistent with results obtained for the *Sulfolobus shibatae* CCA-adding enzyme (a Class I enzyme), where replacing the aspartic acid at position 106 with alanine decreased ATP but not CTP addition (Yue *et al.*, 1998), suggesting that a third carboxylate is not required for catalysis but may play a role in nucleotide binding (Yue *et al.*, 1998). When our study was initiated, the roles of

the DXD or RXD carboxylates of eukaryotic Class II CCA-adding enzymes had not been investigated, neither had any specific role of those amino acids been studied *in vivo* in any organism.

In *Escherichia coli*, where the CCA sequence is encoded by each tRNA gene (Sprinzl *et al.*, 1998), tRNA nucleotidyltransferase serves to repair damaged CCA termini (Zhu and Deutscher, 1987). In many organisms (most eukaryotes and archaeobacteria and some eubacteria) some or all of the tRNA genes do not encode the CCA sequence (Sprinzl *et al.*, 1998) and tRNA nucleotidyltransferase is essential. Indeed, in eukaryotes this enzyme functions in every cellular compartment in which tRNAs are synthesized (nucleus, mitochondrion, chloroplast) as well as in the cytoplasm, where repair of damaged CCA termini occurs (Rosset and Monier, 1965). In *S. cerevisiae*, tRNA nucleotidyltransferase is required for viability (Aebi *et al.*, 1990) and both the mitochondrial and nuclear/cytosolic forms of the enzyme are produced from a single nuclear gene (Chen *et al.*, 1992). Multiple transcriptional and three translational start sites (Chen *et al.*, 1992; Wolfe *et al.*, 1994) give rise to three forms of the enzyme. The largest form is targeted mainly to mitochondria, while the two smaller forms are targeted to the nucleus and the cytosol (Wolfe *et al.*, 1994, 1996).

We are interested in mitochondrial biogenesis and the evolution of sorting isozymes. We have been studying tRNA nucleotidyltransferase genes from yeasts (other than *S. cerevisiae*) and plants, see whether these genes also encode multiple forms of the enzyme that are destined for different cellular compartments. Based on nuclear DNA sequence comparisons (van de Peer *et al.*, 1992), *C. glabrata* is a close relative of *S. cerevisiae*. However, in contrast to the high level of similarity of their nuclear genomes, the mitochondrial genomes of *C. glabrata* and *S. cerevisiae* have some major differences. For example, the mitochondrial genome of *C. glabrata* (19 kbp) is only about 25% the size of the *S. cerevisiae* mitochondrial genome (Clark-Walker and Weiller, 1994). Also, the mitochondrially encoded RNase P RNA of *C. glabrata* is less than half the size of its *S. cerevisiae* counterpart (Shu *et al.*, 1991). While extensive research has been done on mitochondrial protein import in *S. cerevisiae* (Neupert, 1997), little is known about how proteins synthesized on cytoplasmic ribosomes are targeted to mitochondria in *C. glabrata*.

We have used complementation of a temperature-sensitive mutation in *S. cerevisiae* to isolate the *C. glabrata* gene encoding tRNA nucleotidyltransferase. Sequence analysis revealed an open reading frame that could encode a protein of 543 amino acids with 60% identity to tRNA nucleotidyltransferase from *S. cerevisiae*. Results of *in vitro* mutagenesis and plasmid shuffling experiments confirm *in vivo* the essential role of the carboxylates of the DXD and RRD motifs. As in *S. cerevisiae*, the predicted protein contains an amino-terminal extension that is required for mitochondrial targeting. *C. glabrata* CCA1 contains multiple transcriptional start sites, such that proteins produced from the largest transcripts most likely contain the N-terminal mitochondrial targeting sequence. Proteins produced from transcripts initiating between the two start codons would lack the mitochondrial targeting sequence. Our data also suggest that the relative abundance of transcripts depends on the carbon source of the growth medium. More short transcripts, capable of coding for proteins lacking mitochondrial targeting sequences, were seen in RNA isolated from cells grown on glucose than from cells grown on non-fermentable carbon sources. In contrast, more long transcripts (potentially encoding proteins with mitochondrial targeting signals) were detected in RNA isolated from cells grown on non-fermentable carbon sources.

## Materials and methods

### Strains, plasmids and media

*E. coli* strains used were CJ236 (Kunkel *et al.*, 1987), JM105 (Yanisch-Perron *et al.*, 1985) and XL2-blue (Stratagene). *Saccharomyces cerevisiae* strain NT33-5 (relevant genotype *cca1-1 ura3*) is described in Shanmugam *et al.* (1996). Bacteriophage M13mp19 (Yanisch-Perron *et al.*, 1985) was used for mutagenesis. *C. glabrata* strains were CgHTUA (Stoyan *et al.*, 2001) and CBS138 (Clark-Walker and Sriprakash, 1983). Plasmids pRS313 and pRS316 are described in Sikorski and Heiter (1989) and pBluescript II KS<sup>+</sup> (BSKS) was obtained from Stratagene. Plasmid CCA426 (Wolfe *et al.*, 1994), containing the *S. cerevisiae* CCA1 gene, was provided by N. C. Martin. Plasmid USN 3-4, was constructed by transferring the CCA1 gene and its flanking regions on a

*Bam*HI–*Sal*I fragment from plasmid CCA426 into plasmid pRS316. Plasmid pKEH 3-2 was constructed by transferring a *Pvu*II fragment containing a truncated *K. lactis* CCA1 gene under control of the *S. cerevisiae* GPD promoter from plasmid pKE (Deng *et al.*, 2000) into *Pvu*II-cleaved pRS313. *E. coli* transformants were cultivated on LB medium containing 50 µg/ml ampicillin. Strain CJ236 was grown on LB medium containing 15 µg/ml chloramphenicol. Yeast rich medium contained 2% peptone, 1% yeast extract and either 2% glucose (YPD) or 2% glycerol plus 2% ethanol (YPEG). FOA medium (Boeke *et al.*, 1984) contained 0.5 g/l 5-fluoro-orotic acid and was supplemented with 20 mg/l histidine, adenine and tryptophan. Yeast transformants were selected on synthetic complete (SC) medium lacking the appropriate nutrients (Sherman, 1991).

### Nucleic acid purification

The SpinPrep<sup>™</sup> Plasmid Kit (Novagen) was used to isolate plasmid DNA from *E. coli*. Plasmid DNA was isolated from yeast by the method of Ward (1990). Single-stranded and replicative forms of phage DNA were prepared as described by Sambrook *et al.* (1989). Yeast genomic DNA was isolated by the procedure of Hoffman and Winston (1987). The RNeasy Mini Kit (Quiagen) was used to extract RNA from yeast cultures grown to OD<sub>640</sub> = 0.8 in YPD or YPEG medium.

### Isolation of the CCA1 gene

A *C. glabrata* genomic library (Hanic-Joyce and Joyce, 1998) was used to transform (Schiestl and Gietz, 1989) *S. cerevisiae* strain NT33-5 bearing the *cca1-1* mutation. The  $2.9 \times 10^5$  Ura<sup>+</sup> transformants obtained were screened for the ability to grow on YPD at the non-permissive temperature of 37 °C. Plasmid loss analysis was performed on several of the 75 temperature-resistant transformants. Single colony isolates of yeast transformants were inoculated into 20 ml YPD liquid medium and grown to stationary phase at 21 °C. The culture was diluted and plated on YPD medium at 21 °C and the resulting colonies replica-plated to SC medium and SC medium lacking uracil at 21 °C and to YPD medium at 21 °C and 37 °C. Plasmid DNA was isolated from one of the temperature-resistant transformants that exhibited concomitant loss of uracil-prototrophy and

temperature-resistance. When this plasmid, tr71-1, was used to transform strain NT33-5 all of the transformants were temperature-resistant. A 2734 bp *SpeI*–*NheI* fragment from the 6 kbp insert in plasmid tr71-1, which contained the *CCA1* gene and flanking sequences, was inserted into pRS313 and pRS316 to create plasmids pGHC4-4 and pWG1798, respectively.

### Mutagenesis

For mutagenesis of the first in-frame ATG (position 796), a *SpeI*–*BamHI* fragment (positions 489–836) was inserted into BSKS and subsequently transferred to M13mp19 on a *SacI*–*BamHI* fragment. Mutagenesis of the M13 mp19 derivative was performed by the method of Kunkel *et al.* (1987). The oligonucleotide used to change the first ATG to CTG was 5'-GAAACACAGCTGTTCAA-GGC-3'. The insert containing the mutation was excised with *EcoRI* and *BamHI*, and ligated, together with the 378 bp *BamHI* and 2009 bp *BamHI*–*NheI* fragments of tr71-1, into *EcoRI*–*SpeI*-cleaved pRS316 or pRS313 to create plasmids pMT3-2 and GHC42-7, respectively.

Complementary oligonucleotide pairs and the QuikChange™ site-directed mutagenesis kit (Stratagene) were used to change aspartic acid codons. The template for mutagenesis was pGHC4-4. D65 was changed to Ala using 5'-CTGGGCCAAGGAT-CCCACGCCCTGGACATC-3' and 5'-GATGTCC-AGGGCGTGGGATCCTTGGCCCAAG-3' and to Glu using 5'-CCAAGGTTCCACGAGCTCGAC-ATCGCCATC-3' and 5'-GATGGCGATGTCGA-GCTCGTGGGAACCTTGG-3'. D67 was changed to Ala using 5'-CCCACGACCTGGCAATCGCG-ATCAACATCATGTCCG-3' and 5'-CGGACATG-ATGTTGATCGCGATTGCCAGGTCGTGGG-3' and to Glu using 5'-GGTTCACGACCTCGA-GATCGCCATCAACATC-3' and 5'-GATGTTG-ATGGCGATCTCGAGGTCGTGGGAACC-3'. D 157 was changed to Ala using 5'-GAAGACGCT-CTTCGACGGGCTGCCACGCTCAATG-3' and 5'-CATTGAGCGTGGCAGCCCGTCGAAGAGC-GTCTTC-3' and to Glu using 5'-AGACGCTCTG-CGCCGGAAGCCACGCTCAATG-3' and 5'-CATTGAGCGTGGCTTCCCGGCGCAGAGCGT-CT-3'.

Deletions of the amino-terminal encoding portions of the *CCA1* gene were produced by PCR.

Primers for the 5'-end, 5'-ATTTCATATGATCCAAT-TGACTGAGAAAG-3', 5'-GACTCATATGGAG-ACCAGGATATGCAAC-3', 5'-CTATCATATGGA-ACCCCTGACGCTGCGG-3' introduced *NdeI* restriction sites containing in-frame initiator codons (underlined) at positions 835, 853 and 919, respectively, and were used with the 3' primer, 5'-GGCTCTCGAGTACCTGATCATCAGG-3' (complementary to 3' flanking sequences 2801–2825) and introducing an *XhoI* cleavage site at position 2816) to amplify products for construction of plasmids GHC23-1, and GHC15-3 and GHC21-1, respectively. PCR was carried out with *Pfu* polymerase using a 3 min hot start, 30 cycles of 94 °C for 30 s, 50 °C (or 56 °C for construction of GHC21-1) for 1 min and 72 °C for 4 min followed by a 10 min extension at 72 °C. Products of amplification were transferred as *NdeI*–*XhoI* restriction fragments into *SpeI*–*XhoI* digested pRS313 together with a *SpeI*–*NdeI* fragment of WG17-98 containing the promoter of *CCA1*.

### Gene deletion and replacement

A *C. glabrata* strain, GCP1-2, in which sequences of the *CCA1* gene encoding amino acids 1–476 were deleted and replaced with the *S. cerevisiae TRP1* gene, was constructed by integrative transformation. An *SpeI*–*NdeI* fragment (nucleotides 489–794) containing the 5' non-coding region of *C. glabrata CCA1* and filled in at the *NdeI* end using the Klenow fragment of DNA polymerase, was ligated to pBluescript KSII<sup>+</sup> digested with *SpeI* and *SmaI*. A *KpnI* fragment containing a portion of the 3' end of the *CCA1* gene and flanking non-coding sequences (positions 2224–2959) was inserted at the *KpnI* site within pBluescript sequences. A *BglII* fragment derived from plasmid pCXJ11 (Chen, 1996) containing the *S. cerevisiae TRP1* gene was inserted between the two *CCA1*-derived inserts at the *EcoRI* site of pBluescriptIIKS<sup>+</sup> sequences. The resulting plasmid, TCD3, was digested with *SpeI* and *NdeI* (positions 489 and 2835) to liberate a 1.8 kbp fragment that was purified and used to transform strain RCG2, a *CgHTUA* derivative bearing plasmid USN3-4, which carries the *S. cerevisiae CCA1* gene. Transformants were selected on SC medium lacking tryptophan and uracil. Replacement of *CCA1* sequences with *TRP1* sequences in the resulting strain, GCP1-2, was confirmed by the polymerase chain reaction (PCR). To replace

the mutant locus ( $\Delta cca1-1,476::TRP1$ ) in strain GCP1-2, fragments containing *CCA1* constructs were excised from plasmids GHC4-4, MT2-36 and GHC23-1 and introduced by transformation into GCP1-2. The products of transformation were diluted in 10 ml YPD medium and grown to stationary phase at room temperature to allow dilution of the *URA3* gene product. The cells were spread on FOA-containing medium and the resulting FOA-resistant cells were replica-plated to SC medium lacking tryptophan to screen for replacement of the  $\Delta cca1-1-476::TRP1$  locus. Gene replacement and gene identities were confirmed by PCR followed by restriction analysis of amplified products.

#### Plasmid shuffling

Strain GCP1-2 was transformed with pRS313 derivatives containing wild-type or mutant *CCA1* genes. Transformants were selected on synthetic medium lacking histidine and uracil. FOA medium was used to select His<sup>+</sup> Ura<sup>-</sup> cells.

#### DNA sequencing and computer analysis

Sequencing was performed with Sequenase version 2 (Amersham) and [ $\alpha^{35}$ S]dATP or at the Centre for Structural and Functional Genomics, Concordia University, Montreal. Overlapping deletion clones were created using the Erase-a-base system (Promega). Sequence alignments, computer modelling and predictions were carried out using the software provided with PCGENE and through GenBank.

#### Transcript mapping

Sequences of the 5' ends of *CCA1* primary transcripts were determined using the RLM-Race kit (Ambion) with 10  $\mu$ g total RNA isolated from strain CBS138. The inner primer 5'-GGATGCTATGTGGTTTCACACCGT-3' (complementary to nucleotides 1073–1096), and the outer primer 5'-AACAGCTTTGTGGTAGCGGTCTCA-3' (complementary to nucleotides 1134–1157), were used, along with inner and outer RNA adapter primers, in RT-PCR reactions with *Taq* DNA polymerase (MBI Fermentas). Amplifications were performed with a hot start, and 35 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s,

followed by an extension at 72 °C for 7 min. Products of RT-PCR reactions were extracted from agarose gels, ligated to *EcoRV*-digested pBlue-script (T-tailed using *Taq* DNA polymerase and dTTP at 72 °C) and sequenced.

## Results and discussion

We used complementation of a temperature-sensitive mutation (*cca1-1*) in *S. cerevisiae* to isolate, from a *C. glabrata* genomic library, a gene encoding tRNA nucleotidyltransferase. A portion of the 6 kbp insert in the complementing plasmid, tr71-1, was sequenced and shown to contain an open reading frame of 1629 bp that extended from an ATG at position 796 to a TAA stop codon at position 2425. The predicted 543 amino acid protein has a high level of identity (60%) with the predicted amino acid sequence of *S. cerevisiae* tRNA nucleotidyltransferase (Figure 1). Indeed, a comparison of amino acid residues 46–257 of the *C. glabrata* enzyme with amino acid residues 60–273 of the *S. cerevisiae* enzyme revealed 80% identity. This amino-terminal region of the *C. glabrata* enzyme contains the active site signature of the nucleotidyltransferase superfamily (Holm and Sander, 1995). The extended  $\alpha$ -helix in this region groups this protein in the Class II subfamily (Yue *et al.*, 1996), which includes other eubacterial and eukaryotic tRNA nucleotidyltransferases and eubacterial poly(A) polymerase.

While Class II enzymes share a conserved amino-terminal domain, their carboxy-terminal regions are more divergent (Shanmugam *et al.*, 1996; Yue *et al.*, 1996; Martin and Keller, 1996). In keeping with this observation, the *C. glabrata* and *S. cerevisiae* CCA-adding enzymes share only 44% identity over their carboxy-terminal regions (amino acids 258–543 of the *C. glabrata* protein and amino acids 247–546 of the *S. cerevisiae* protein, Figure 1). The finding of an RNA-binding site in the carboxy terminus of *E. coli* poly(A) polymerase I and the lack of sequence similarity between the carboxy-terminal regions of poly(A) polymerase and CCA-adding enzymes led to the proposal that the C-terminal region of Class II enzymes confers substrate specificity (Raynal and Carpousis, 1999).

For the Class II enzyme, *E. coli* poly(A) polymerase I, five essential carboxylates, including the three carboxylates of the DXD and RRD motifs,

have been defined (Raynal and Carpousis, 1999). These DXD and RRD are characteristic of Class II nucleotidyltransferases (Yue *et al.*, 1996) and are conserved in the *C. glabrata* CCA-adding enzyme (Figure 1). To investigate *in vivo* the importance of these two motifs, we used mutagenesis and plasmid shuffling. The codons D65 and D67 (in the DXD motif) and D157 (in the RRD motif) were altered to alanine or glutamate and the mutant genes were introduced, as pRS313 derivatives, into a *C. glabrata* strain (GCP1-2) containing a plasmid-borne wild-type *CCA1* gene from *S. cerevisiae* and a mutant chromosomal *CCA1* gene ( $\Delta cca1-1-476::TRP1$ ) in which sequences encoding amino acids 1–476 were replaced with the *S. cerevisiae* *TRP1* gene. Selection of cells that retained only plasmids bearing the mutant *CCA1* genes was accomplished on FOA-containing medium. When any of these aspartates were substituted with alanine the cells were not viable (Figure 2). This demonstrates that the carboxylates of the DXD and RRD motifs (Yue *et al.*, 1996) play an essential role in eukaryotic tRNA nucleotidyltransferase *in vivo*. This is in good agreement with *in vitro* studies of the *Sulfolobus* (Yue *et al.*, 1998) and *E. coli* (Seth *et al.*, 2002) CCA-adding enzymes. In both of these cases converting the aspartates corresponding to positions D65 and D67 of the *C. glabrata* enzyme to alanine eliminated incorporation of both CTP and ATP. Also, in both the *Sulfolobus* and *E. coli* enzymes an aspartic acid-to-alanine substitution at the position corresponding to D157 in the *C. glabrata* enzyme severely impaired the addition of ATP, but not CTP (Yue *et al.*, 1998; Seth *et al.*, 2002). Our *in vivo* results confirm the essential role of these three carboxylates.

While the conservative mutations, D65E or D157E, did not significantly affect cell growth, the D67E mutation was lethal (Figure 2). This suggests that the length of the side chain of the residue at position 67 also is critical for enzyme activity. A requirement for aspartic acid at this position has been observed *in vitro* with the Class I CCA-adding

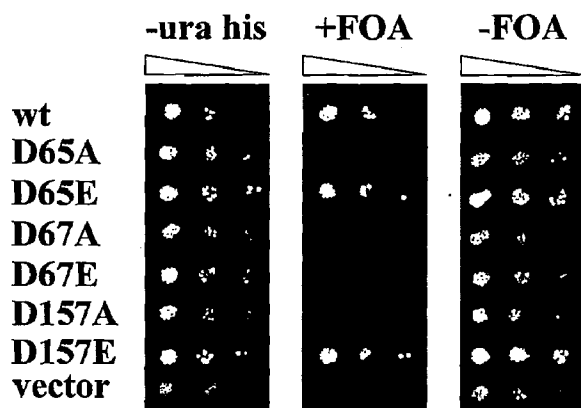
enzyme from *Sulfolobus*, where the corresponding D55E substitution resulted in a 300- and 100-fold reduction of ATP and CTP addition, respectively (Yue *et al.*, 1998). If the D67E mutation in *C. glabrata* produces a similar decrease in enzyme activity, it is not surprising that these cells cannot survive as tRNA nucleotidyltransferase levels in yeast are low (Chen *et al.*, 1990). In contrast, the viability of the D65E mutant is not unexpected, since DNA sequence from *Archaeoglobus fulgidus* (Klenk *et al.*, 1997) indicates that its CCA-adding enzyme will have a glutamic acid at the corresponding position.

Eukaryotic tRNA nucleotidyltransferases have an amino-terminal extension not found in their bacterial counterparts (Aebi *et al.*, 1990; Shanmugam *et al.*, 1996; Deng *et al.*, 2000; Nagaike *et al.*, 2001; Reichert *et al.*, 2001). Previous studies have shown that gene deletions that cause complete (Deng *et al.*, 2000) or partial removal of this extension (Chen *et al.*, 1992), while precluding mitochondrial targeting, do not affect cytosolic/nuclear enzyme activity. The *C. glabrata* gene encodes a 44 amino acid extension that does not align with the protein from *E. coli* and that could encode a mitochondrial targeting signal (Figure 1). Indeed, the N-terminal 18 amino acids of the *C. glabrata* protein can be arranged in a helical wheel configuration that shows a hydrophobic face and a basic and hydroxylated face characteristic of a mitochondrial targeting signal (von Heijne *et al.*, 1989). The extension also contains a potential mitochondrial processing peptidase site (Gavel and von Heijne, 1990) after amino acid 15. In fact, the amino-terminus of the *C. glabrata* protein more clearly resembles a mitochondrial targeting signal than does the corresponding region of the *S. cerevisiae* protein. Evidence that *C. glabrata* tRNA nucleotidyltransferase is targeted to mitochondria is provided by the finding that the *C. glabrata* *CCA1* gene also complements the *cca1-1* mutation in *S. cerevisiae* on non-fermentable carbon sources (Figure 3).

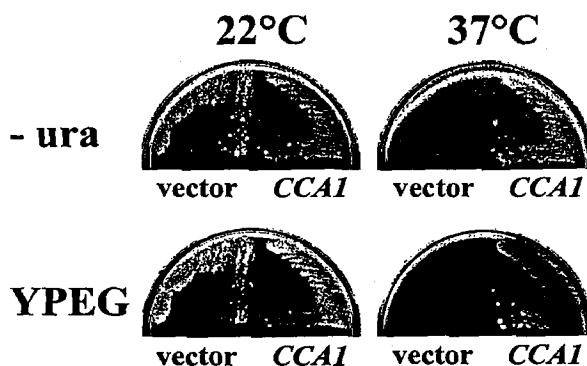
**Figure 1.** Alignment of the predicted amino acid sequences of *Candida glabrata* (CGCCA), *Saccharomyces cerevisiae* (SCCCA), and *Escherichia coli* (ECCCA) tRNA nucleotidyltransferases. Standard one letter abbreviations for amino acids are used. (\*) indicates a position that is perfectly conserved; (·) indicates a position that is well-conserved; (–) indicates a gap introduced to optimize alignments. The conserved DXD and RRD motifs are shown in bold. Numbers indicate position in each protein sequence

CGCCA	MFKAIRRVFTMI-----PRIQLTEKETRICNLLKDYTAHYN-SLH	39
SCCCA	MLRSTISLLMNSAAQKMTNSNFVLNAPKITLTKVEQNICNLLNDYTDLYNQKYH	55
ECCCA	-----	
CGCCA	YGQEPLTLRITGGWVRDKLLGQGSHDLDIAINIMSGEEFANGLNGYLLEHFDKYG	94
SCCCA	NKPEPLTLRITGGWVRDKLLGQGSHDLDIAINVMSGEQFATGLNEYLQQHYAKYG	110
ECCCA	-----MKIYLVGGAVRDALLGLPVKDRDWVVVGSTPQEMLDAGYQQVGRDFPVFL	50
	... ..** *** **	
CGCCA	VKPHSIHKIDKNPEKSKHLETATTKLFDVEVDFVNLRSEEYTEDSRIPPTTQFGTP	149
SCCCA	AKPHNIHKIDKNPEKSKHLETATTKLFGVEVDFVNLRSEKYTELSRIPKVCFGTP	165
ECCCA	HPQTHEEYALARTERKSGSGYTGTCTCYAAPD-----VTL	84
	..*....	
CGCCA	EEDALRRDATLNALFYNIQQDAVEDFTKRGWQDLQDGVLRTPPLPARQTFLLDDPLR	204
SCCCA	EEDALRRDATLNALFYNIHKGEVEDFTKRGWQDLQDGVLRTPPLPAKQTFLLDDPLR	220
ECCCA	EDDLKRRDLTINALAQDDNGEIDPYN--GLGDLQNRLLRHVSPA--FGEDPLR	134
	*.* *** *.*** . . . . . * * . . . ** ** * .****	
CGCCA	VLRLIRFASRFN---FNIEAGVLKEMHDPEINEAFNNKISRERIGVEMEKILVGP	256
SCCCA	VLRLIRFASRFN---FTIDPEVMAEMGDPQINVAFNSKISRERIGVEMEKILVGP	272
ECCCA	VLRVARFAARYAHLGFRIADETLALMREMTAGELEH-LTPERVWKETESALTTR	188
	***. ***.*. . * . . . . * . . . . .***. * . * . .	
CGCCA	NPILGLKLIQRTHLENIIFLWHGDQSVIEYNRNKWPQTKDVEDIYKKGIFNHHLK	311
SCCCA	TPLLALQLIQRAHLENIIFFWHNDSSVVKFNEENCQDMDKINHVVNDNIINSHLK	327
ECCCA	NPQVFFQVLRDCGALRVLFPEIDALFGVPAPAKWHPEIDTGIHTLMTLSMAAMLS	243
	. * . . . . . . * . . . . . . . . . . . . . . *	
CGCCA	NFIHH-YKDFLSRYLKLKRAIETKDKSFQQNFLLASILIPMADLKIIRLPKKKLN	365
SCCCA	SFIEL-YPMFLEKLPILEKIG-RSPGFQQNFILSAILSPMANLQIIGNPKKKIN	380
ECCCA	PQVDVRFATLCHDLGKGLTPPELWPRHHGHGPAGVKLVEQLCQRLRVPNEIRDLA	298
	. . . . . . . . . . . . . . . . . . . . . . . . .	
CGCCA	NTPVSESIIVREGLKFNKASSIVDARCVENIAAYNSMVEKYLQSGDLKRSEVGTG	420
SCCCA	NLVSVTESIVKEGLKLSKNDAAVIAKTVDSICSYEELAKFADRSQKKSEIGIF	435
ECCCA	RLVAEFHDLIHTFPMLNPKTIVKLFDSIDAWRKPRVEQLALTSEADVGRGTGF-	352
	. . . . . . . . . . . . . . . . . . . . . . . *	
CGCCA	LRRLRGDWEIVHYVSLMDQYLKYISRKDNVNIID-KYDRFWNYIQEQNLQSDSK	474
SCCCA	LRNFGEWETAHFASLSDAFLKIPKLETKKIELLFQNYNEFYSYIFDNNLNNCHE	490
ECCCA	-----ESADYPQGRWLREAW	368
	. * . . * . . .	
CGCCA	MVPIIDGKRMVKILETKPGPWIGKINDEVILWQFDHPQGTEQELISLIKSILPNY	529
SCCCA	LKPIVDGKQMAKLLQMKPGPWLGKINNEAIRWQFDNPTGTDQELITHLKAILPKY	545
ECCCA	VAQSVPTKAVV-----EAGFKGVEIREELTRRRRIAASWKEQRCPKPE-----	412
	. . . . * . . . . * . . . . * . . . . . . . . . .	
CGCCA	LQCYMSAIYRFQIY	543
SCCCA	L-----	546
ECCCA	-----	412

Figure 1



**Figure 2.** Growth characteristics of *cca1* mutants revealed by plasmid shuffling. Strain GCP1-2 was transformed with pRS313-derivatives encoding wild-type (wt) or altered tRNA nucleotidyltransferases (amino-acid substitutions indicated) or with pRS313 (vector). Transformants were grown to mid-log phase in SC medium lacking uracil and histidine. Equal numbers of cells then were diluted serially ten-fold and spotted on SC medium lacking uracil and histidine (–ura his) and FOA medium containing (+FOA) or lacking (–FOA) 5-fluoro-orotic acid. Plates were photographed following incubation at 30 °C for 3 days

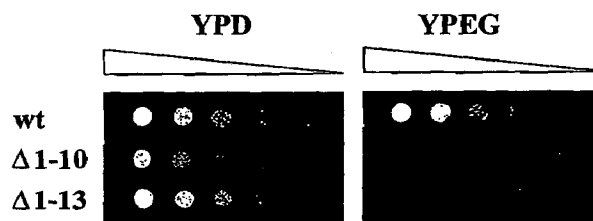


**Figure 3.** Complementation of the *cca1-1* mutation of *S. cerevisiae* by *C. glabrata* sequences. Growth characteristics of *cca1-1* mutant strain NT33-5 transformed with pRS316 (vector) or with pWG17-98 bearing the *CCA1* gene from *C. glabrata* (*CCA1*). Cells growing on SC medium lacking uracil (–ura) at 22 °C were replica-plated to –ura and YPEG media and incubated for 3 days at 22 °C or 37 °C

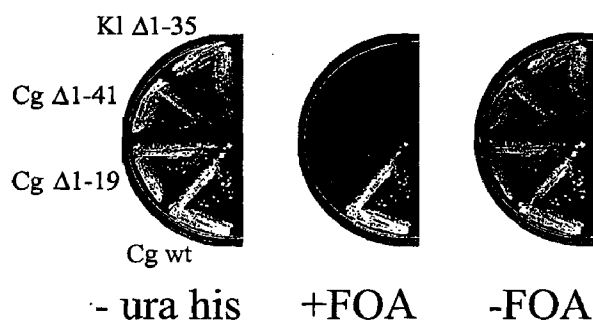
To confirm that N-terminal sequences of *C. glabrata* *CCA1* are involved in mitochondrial targeting, we examined whether N-terminally truncated forms of the *CCA1* gene product could support growth on a non-fermentable carbon source. Using integrative transformation, we replaced the

mutant locus,  $\Delta cca1-1,476::TRP1$ , in strain GCP1-2 with altered forms of *C. glabrata* *CCA1* expressed from the *CCA1* promoter region. FOA-resistant recombinants expressing a shortened version of *CCA1* that lacked the first 10 amino acids were viable in glucose-containing medium but were unable to grow in medium containing glycerol and ethanol as the carbon sources (Figure 4). This result indicates that the first 10 amino acids of *CCA1* comprise part of a mitochondrial targeting signal. A similar result was observed when a recombinant lacking the first 13 amino acids and bearing an R14M substitution was generated (Figure 4). Taken together, these data indicate that the first 10 amino acids are necessary to direct this enzyme to the mitochondrion, but that the first 13 amino acids are not required for enzyme activity.

We used plasmid shuffling to demonstrate that more extensive deletions of the N-terminal extension were lethal. After selecting transformants of strain GCP1-2 ( $\Delta cca1-1,476::TRP1$ ) which carried both our truncated gene of interest (carried on pRS313) and a rescue plasmid containing the *S. cerevisiae* *CCA1* gene, transformants were replica-plated to FOA-containing medium to assess the effect of loss of the rescue plasmid. No growth was observed when the encoded protein lacked either the first 19 amino acids and contained a K20M substitution or lacked the first 41 amino acids and contained a Q42M substitution (Figure 5). Therefore, while the first 13 amino acids of the *C. glabrata* *CCA*-adding enzyme are not required for enzyme function, at least part of the remainder of



**Figure 4.** Growth characteristics of recombinants. Strain GCP1-2 was transformed with fragments containing genes encoding wild-type (wt) or truncated tRNA nucleotidyltransferase lacking the first 10 or 13 amino acid residues ( $\Delta 1-10$ ,  $\Delta 1-13$ , respectively). Recombinants were grown to mid-log phase in YPD. Equal numbers of cells then were diluted serially ten-fold and spotted on YPD and YPEG medium. Plates were photographed after 2 days incubation at 30 °C



**Figure 5.** Effect on cell growth of deletions of amino-terminal coding sequences. Strain GCPI-2 was transformed with pRS313 derivatives bearing wild-type (wt) or truncated *CCA1* genes from *C. glabrata* (Cg) or *K. lactis* (Kl). Truncated genes encoded proteins lacking the first 19 ( $\Delta 1-19$ ), 41 ( $\Delta 1-41$ ) or 35 ( $\Delta 1-35$ ) amino acids. Transformants were grown on SC medium lacking histidine and uracil ( $-ura\ his$ ) and were replica-plated to FOA medium containing (+FOA) or lacking ( $-FOA$ ) 5-fluoro-orotic acid. Plates were incubated for 3 days at 30°C

the N-terminal extension is required. This observation was unexpected as these N-terminal amino acids represent an extension not found in the *E. coli* enzyme (see Figure 1) and might not be expected to play a role in CCA addition. Also, we previously showed that complete removal of the 35 amino acid N-terminal extension of the *K. lactis* CCA-adding enzyme eliminated growth on non-fermentable carbon sources but did not affect nuclear/cytosolic activity when assessed by complementation in *S. cerevisiae* (Deng *et al.*, 2000). Our findings suggest an additional role for the N-terminal amino acids in the *C. glabrata* enzyme that is not required for the *K. lactis* enzyme. However, we also observed that the truncated *K. lactis* gene product did not complement the  $\Delta cca1-1,476::TRP1$  mutation in *C. glabrata* (Figure 4) and that the  $\Delta 1-19$  and  $\Delta 1-41$  truncated *C. glabrata* gene products failed to complement the *cca1-1* and  $\Delta cca1::HIS3$  mutations in *S. cerevisiae* (data not shown). The potential role of the N-terminal amino acids of the *C. glabrata* CCA-adding enzyme remains to be elucidated as do the differences in the ability of the truncated *K. lactis* gene product to function in *S. cerevisiae* but not in *C. glabrata*.

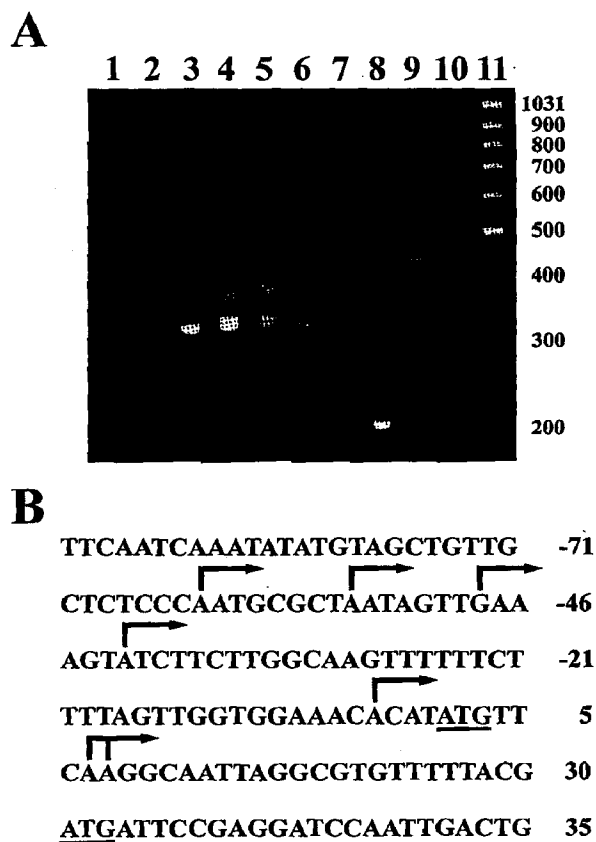
Interestingly, we found that the mitochondrial phenotype of N-terminal truncations was dependent on expression levels. Plasmid pRS316, while single copy in *S. cerevisiae*, replicates with high copy number in *C. glabrata* attaining 10–30 copies/cell

(Zhou *et al.*, 1994). N-terminal truncations of *C. glabrata* *CCA1* that lacked the first 10 or 13 amino acids and were borne on a related plasmid, pRS313, conferred growth on a non-fermentable carbon source. In contrast, as discussed previously, as single copy chromosomal genes these N-terminal truncated forms did not allow growth on non-fermentable carbon sources. The effects of different levels of expression were the same when the *C. glabrata* genes were expressed in the *S. cerevisiae* *cca1-1* mutant. At low copy (as pRS315 derivatives), complementation of the mitochondrial defect did not occur, while in high copy complementation of the mitochondrial defect was observed (data not shown). The fact that even at low copy number these proteins can complement the nuclear/cytosolic defect suggests that enough of the protein is being made to carry out nucleotide addition. Redundant mitochondrial targeting signals have been found in other proteins (e.g. Bedwell *et al.*, 1987). When the first nine amino acids were removed from the *S. cerevisiae* tRNA nucleotidyltransferase, the resulting cells were still able to grow on a non-fermentable carbon source, although mitochondrial CCA-adding enzyme levels were reduced to about 10% of wild-type levels (Chen *et al.*, 1992). Complementation of the *cca1-1* mutation occurred even when the truncated *S. cerevisiae* *CCA1* gene was carried in low copy number on a centromeric vector. Perhaps a similar situation exists here and an inefficient mitochondrial targeting signal is present elsewhere in the *C. glabrata* CCA protein. If additional targeting information does exist downstream of amino acid 10 in the *C. glabrata* protein, it must function inefficiently, as it does not complement the mitochondrial defect unless it is present in excess. It is also possible that no additional specific mitochondrial targeting information exists on this protein and, simply by having an excess of this enzyme in the cytosol a small amount (sufficient to complement the mitochondrial defect), gets into the mitochondrion. However, when we overexpressed the lupin CCA-adding enzyme in *S. cerevisiae* we found no complementation of the mitochondrial defect (Shanmugam *et al.*, 1996), suggesting that simply having excess cytosolic tRNA nucleotidyltransferase is not sufficient for entry into mitochondria.

Multiple transcription start sites are commonly used to produce protein products with more than one cellular destination (see Small *et al.*, 1998,

for review). Within the *S. cerevisiae* *CCA1* gene there are three in-frame start codons (Aebi *et al.*, 1990). Transcription start sites have been mapped upstream of and between the first two start codons (Wolfe *et al.*, 1994). The longest form of the protein is targeted primarily to mitochondria, while the shorter forms of the protein localize to the cytosol and nucleus (Wolfe *et al.*, 1994, 1996). We investigated whether multiple transcription start sites also are used to produce different forms of the *CCA1* protein in *C. glabrata*. Using RLM-RACE, multiple PCR products were observed upon amplification of cDNA ends from RNA extracted from cells grown in glucose-containing medium (Figure 6). Sequencing of these products revealed that transcription termini mapped to positions -63, -55, -48, -4, +7 and +8 of the *C. glabrata* *CCA1* gene (Figure 6). The longer transcripts initiating from positions -63 to -4 contain the first AUG and could give rise to a protein with an N-terminal mitochondrial targeting sequence or, if the second AUG is used, proteins without a mitochondrial targeting sequence. Two initiation sites (at +7 and +8) are downstream of the first AUG and, therefore, translation of these shorter messages cannot initiate prior to the second in-frame AUG. The next in-frame AUG coding for M73 is probably not used for translation because it is downstream of the DXD motif and, as shown above, engineered products lacking the 19 N-terminal amino acids and containing a K20M substitution are not functional. The transcript initiated from position -4 has a short leader and this may affect the amount of translation that begins with the first AUG and may lead to translation initiating from the second AUG. As yet it is not known whether *C. glabrata* has a preferred context for AUG codons. While translation of *S. cerevisiae* mRNAs does not require a leader sequence (Maicas *et al.*, 1990), initiation is less efficient for mRNAs with short leaders (van den Heuvel *et al.*, 1989, Cigan and Donahue, 1987). Kozak (1991) demonstrated *in vitro* that, as leader length decreases, translation initiation at a second downstream AUG increases. Another possibility is that either AUG can be used to initiate translation from certain transcripts. Some bifunctional transcripts, in which translation can initiate from either AUG, thereby producing sorting isozymes, have been observed for the *S. cerevisiae* *MOD5* gene (Slusher *et al.*, 1991). Assuming that the first AUG can be used for translation of the *C. glabrata*

CCA enzyme, differential use of transcription initiation sites alone would be sufficient to generate both a long form of the enzyme that has a mitochondrial targeting sequence and a short form that lacks it.



**Figure 6.** (A) Products of RLM-RACE reactions. Total RNA was isolated from strain CBS138 grown in YPD (lanes 1–4, 9) or YPEG (lanes 5–8, 10) and used in RLM-RACE reactions. Products of inner PCR reactions (5  $\mu$ l aliquots) were separated by electrophoresis in a 2% agarose gel. Primer pairs used for PCR were inner *CCA1*-specific primer and inner RNA adapter primer (lanes 1–8) or inner *RPSO*-specific primer and inner RNA adapter primer (lanes 9 and 10). Controls included reactions lacking tobacco-acid pyrophosphatase treatment (lanes 1 and 8) and lacking reverse-transcription (lanes 2 and 7). For comparative purposes, results for two different RNA preparations from YPD- (lanes 3 and 4) and YPEG-grown cells (lanes 5 and 6) are shown. The 5'-termini of inner PCR products contain 36 bp of RNA adapter sequence. Size markers (lane 11) are the GeneRuler™ 100 bp DNA ladder from MBI Fermentas. (B) Positions of transcription initiation sites. DNA sequence (nucleotides 701–850, GenBank Accession No. AF098803) immediately upstream and downstream of the first two ATG codons (underlined) is shown. Each transcription initiation site is indicated by an arrow above the initiating nucleotide

The use of multiple transcription start sites to generate *CCA1* sorting isozymes is not a universal mechanism. The *CCA1* gene from the petite-negative yeast, *K. lactis*, does not have an in-frame ATG immediately downstream of the N-terminal mitochondrial targeting sequence information (Deng *et al.*, 2000). The first ATG is separated from the second in-frame ATG by only two amino acids and sequences required for mitochondrial import are downstream of the second ATG. Therefore, it is likely that only one protein is produced from the *K. lactis CCA1* gene. In this case the protein always contains sequences for mitochondrial import, which presumably are inefficient and target only some of the protein to the mitochondrion. Indeed, the shorter form of the yeast *TRM1* gene product contains an inefficient mitochondrial targeting signal, such that this protein is shared between the nucleus/cytosol and the mitochondrion (Ellis *et al.*, 1987). Alternatively, all of the gene product may be targeted to mitochondria and processed before being distributed back to the cytosol, as has been proposed for the yeast *FUM1* gene product (Sass *et al.*, 2001). Since *K. lactis* requires mitochondrial protein synthesis for viability (Murray *et al.*, 2000), having a single form of CCA-adding enzyme that is targeted to multiple compartments may help to coordinate cytoplasmic and mitochondrial translation.

When it became evident that multiple transcription start sites were used for expression of the *C. glabrata CCA1* gene, we investigated whether transcription start site selection could be affected by carbon source. Because PCR reactions select for templates with the highest amplification rates within a reaction, we can not quantitatively assess the relative levels of transcripts within any single assay (e.g. growth on a specific carbon source). However, we can compare relative differences between transcript levels of products from different carbon sources. Multiple transcription start sites are used both on fermentable and non-fermentable carbon sources, but the relative levels of individual transcripts differ (Figure 6). Cells grown on the non-fermentable carbon source produced more of the longer *CCA1* transcripts (capable of generating the mitochondrial targeting information) and less of the shorter transcripts (lacking this information) compared to cells grown on glucose. To ensure that the observed differences in expression patterns were reproducible, multiple RNA samples were

prepared and analysed from each growth condition and no appreciable differences were seen between replicates (Figure 6, cf. lanes 3 and 4, or 5 and 6). In addition, no change was seen in the sizes or relative abundance of transcripts from another unique copy *C. glabrata* gene, *RPS0* (encoding a ribosomal protein), when cells were grown with the different carbon sources (Figure 6, lanes 9 and 10). The differences in *CCA1* transcript abundances may be due to differential use of transcription start sites or to different stabilities of the transcripts under the altered growth conditions. Perhaps the *CCA1* gene contains promoter elements that are differentially regulated in response to different carbon sources. Levels of transcripts from the *Aspergillus nidulans idpA* gene, coding for forms of NADP-dependent isocitrate dehydrogenase and targeted to different intracellular destinations (mitochondria or cytosol and peroxisomes), have been shown to differ, depending on the carbon source provided (Szewczyk *et al.*, 2001). More research will be required to determine how the differences in *CCA1* transcript levels arise. It will be important to define promoter and regulatory sequences and proteins that may be involved. It is perhaps worth noting that the differential expression pattern demonstrated here for the *CCA1* gene most likely would not be detected in global surveys of gene expression that employ microarrays (e.g. DeRisi *et al.*, 1997), because under both conditions there are multiple transcripts from the *CCA1* gene and only the ratio of small to large transcripts changes.

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